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Article

Accurate quantification of nucleic acids using hypochromicity measurements in conjunction with UV spectrophotometry

Alison Nwokeoji, Peter M Kilby, David Portwood, and Mark J. Dickman

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Accurate quantification of nucleic acids using hypochromicity measurements in

conjunction with UV spectrophotometry

Alison O. Nwokeoji[1], Peter M. Kilby[2], David E. Portwood[2] and Mark J. Dickman[1*]

[1]Department of Chemical and Biological Engineering, ChELSI Institute, Mappin Street, University of Sheffield, S1 3JD, UK

[2]Syngenta, Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK

*corresponding author

Email m.dickman@sheffield.ac.uk

Phone +44 (0)114 22 27541

Fax +44 (0)114 22 27501

Abstract

UV absorbance spectrophotometry is widely used for the quantification of nucleic acids. For accurate quantification it is important to determine the hypochromocity of the oligonucleotide or complex nucleic acid structure. The use of thermal denaturation studies in conjunction with UV spectrophotometry to determine hypochromicity requires prolonged, elevated temperatures, which may cause partial hydrolysis of RNA. In addition, dsRNA is difficult to denature even at elevated temperature and the extinction coefficients of nucleic acids are also affected by temperature, which makes it difficult to accurately determine the nucleic acid concentration. To overcome these caveats, we have utilised the chemical denaturant dimethyl sulfoxide which, in conjunction with a short thermal denaturation prevents renaturation of the duplex nucleic acids (dsDNA/RNA). Using this approach, we have measured the absorbance of both the unstructured and structured nucleic acids to accurately measure their hypochromicity and determine their extinction coefficients. For a range of different dsRNA we have for the first time determined values of 46.18-47.29 µg/ml/A_{260} for the quantification of dsRNA using UV spectrophotometry. Moreover, this approach enables the accurate determination of the relative proportion of duplex nucleic acids in mixed ds/ss nucleic acid solutions, demonstrating significant advantages over current methods.

Keywords:

UV absorbance; hypochromicity, dsRNA, UV spectrophotometry; A_{260} unit

Introduction

UV absorbance spectrophotometry remains one of the most popular methods for the rapid quantification of nucleic acids.[1-3] The absorbance of DNA and RNA at 260 nm is measured and the concentration determined using the Beer Lambert law ($A = \varepsilon.C.l$) in conjunction with the molar extinction coefficient (ε) of constituent nucleotides. However, inaccuracies in the extinction coefficients can reduce the accuracy of results obtained.[4,5] Three common methods are currently used to calculate nucleic acid mass concentration/$A_{260\ nm}$. The first method ignores base composition and assumes that the average molar mass and extinction coefficient of nucleotides is 330 g/mole and 10 mMol^{-1} cm^{-1}, respectively.[6,7] For an absorbance ($A_{260\ nm}$) of 1, a concentration of 33 µg/mL is obtained for single stranded (ss) oligonucleotide using the Beer Lambert equation.[4,6] The second method assumes that ε is the sum of nucleotide extinction coefficients weighted by the number of times each base appears in the sequence.[6] Both methods do not account for potential hypochromicity in the oligonucleotide or complex nucleic acid structures. For accurate quantification it is important to determine the hypochromicity of the oligonucleotide or complex nucleic acid structure. The extinction coefficient of double stranded nucleic acids is less than the sum of the extinction coefficients of the corresponding two single stranded nucleic acids.[8] This hypochromic effect of nucleotides is attributed to dipole induced dipole interactions resulting from base stacking.[9] In addition to using the sum of the nucleotide extinction coefficients for a given sequence a factor of 0.9 is often applied to take into account base stacking interactions in single stranded nucleic acids.[10] The third method described as the near-neighbour calculation attempts to account for hypochromicity.[5] It has been reported to

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yield extinction coefficients within 20% of the experimentally measured extinction coefficients.[4,6]

Hypochromicity measurements can be made by comparing the absorbance of the non-denatured and denatured nucleic acid and determining the melting profile of nucleic acids using UV spectrophotometry in conjunction with thermal denaturation.[11,12] However, at high temperatures, partial hydrolysis of RNA may occur, whereas at moderate temperatures, complete denaturation is not guaranteed. Furthermore very high temperatures are required to denature large dsRNA and the extinction coefficients of nucleic acids are affected by temperature, which makes it difficult to accurately determine the RNA concentration.[13] Alternatively, the hypochromicity and concentration of the RNA or DNA can be measured based on the absorbance of the intact molecule with their corresponding nucleosides generated from hydrolysis or enzymatic reactions. Treatment with 0.3 M NaOH at 37°C or nucleases can be used to achieve nucleic acid hydrolysis.[6,14] The limitation in the NaOH hydrolysis method is that deamination of C to U can potentially occur.[4] Hydrolyses of nucleic acids at neutral pH have been reported; however it requires long incubation at 95 °C.[15] Moreover, potential incomplete hydrolysis of complex and modified nucleic acid structures may result in inaccurate quantification.[6]

 A previous study using NMR to accurately determine extinction coefficient yielded more accurate coefficients for nucleotide monophosphates. [4] The same study looked at the deviations between predicted and measured extinction coefficient and recommended

mass concentration/$A_{260 \text{ nm}}$ coefficients of 37 and 38 µg/ml/$A_{260 \text{ nm}}$ for calculation of ssDNA and ssRNA concentrations, respectively. To our knowledge there is no consensus accurate value for mass concentration/A_{260} unit (µg/mL) of long dsRNA. However, values of 40 µg/mL/A_{260} and 50 µg/mL/ A_{260}, are commonly used for RNA and dsDNA, respectively.[2] Moreover, there is no current method that accurately determines and validates the extinction coefficients/hypochromicity of dsDNA, dsRNA and mixed ds/ss-nucleic acids.[4]

In this study, we have developed a high throughput method for the accurate quantification of nucleic acids using UV spectrophotometry in conjunction with hypochromicity measurements. In addition, our method enables the accurate quantification of duplex nucleic acids in mixed ds/ss nucleic acid solutions demonstrating significant advantages over current methods.

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Experimental Section

Chemicals and Materials

Genes were synthesised by GeneArtGene Synthesis (Invitrogen Life Technologies). Ampicillin sodium salt, tetracycline hydrochloride, isopropyl β-D-1-thiogalactopyranoside (IPTG) ≥99%, sodium dodecyl sulphate (SDS) sodium chloride (NaCl), dimethyl sulfoxide (DMSO), RNA from baker's yeast (*S. cerevisiae*) and phenylalanine specific transfer RNA from brewer's yeast were all obtained from (Sigma-Aldrich, Poole, UK). TRIzol Max, isopropanol and ethanol (ThermoFisher Scientific, NJ, US), were used for nucleic acid purifications. Oligonucleotides were purchased from Eurofins Genomics, Ebersberg, Germany and both transfer RNAs were from Sigma-Aldrich, Poole, UK.

Expression of dsRNA using *E. coli* HT115 (DE3).

The *E. coli* strain, HT115 (DE3) [16] was obtained from Cold Spring Harbor Laboratory, NY, USA. Plasmids pCOIV and pDome11 that contain in-house designed 686 bp and 481 bp sequences respectively, flanked on both sides with T7 promoters were ordered from Gene Art® Gene synthesis (Invitrogen). The *E. coli* HT115 (DE3) cells were transformed with either pCOIV or pDome11. The pCOIV and pDome11 transformed cells were grown in culture and induced with IPTG to express dsRNAs as previously described. [17]

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Analysis of purified dsRNA and total RNA

RNA quantification was determined using a NanoDrop™ 2000c spectrophotometer (ThermoFisher Scientific). RNA concentrations were determined by absorbance at 260 nm. Absorbance data using a NanoDrop™ 2000c spectrophotometer were normalized to a 1.0 cm (10.0 mm) path. The $A_{260/280}$ and $A_{260/230}$ ratios were obtained using the NanoDrop™ instrument. Additional analysis of the RNA was performed using ion-pair reverse phase chromatography using a 10 μL injection from 100 μL of eluted/re-suspended RNA.

Ion Pair-Reverse Phase High Performance Liquid Chromatography (IP-RP HPLC)

Samples were analysed by IP-RP-HPLC on Agilent 1100 series HPLC using a Proswift RP-1S Monolith column (50 mm x 4.6 mm I.D. ThermoFisher). Chromatograms were generated using UV detection at a wavelength of 260 nm. The chromatographic analysis was performed using the following conditions: Buffer A 0.1 M triethylammonium acetate (TEAA) pH 7.0 (Fluka, UK); Buffer B 0.1 M TEAA, pH 7.0 containing 25% acetonitrile (ThermoFisher). RNA was analysed using the following gradients. Gradient (1) starting at 22% buffer B to 27% in 2 minutes, followed by a linear extension to 62% buffer B over 15 minutes, then extended to 73% buffer B over 2.5 minutes at a flow rate of 1.0 ml/min at 50 ℃. Gradient (2) starting at 22% buffer B to 27% in 2 minutes, followed by a linear extension to 72% buffer B over 15 minutes, then extended to 73% buffer B over 2.5 minutes, at a flow rate of 1.0 mL/min at 50 ℃ .

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Determination of nucleic acid hypochromicity

Nucleic acids synthesized *in vitro* and in bacteria were purified using RNASwift as previously described [18] in order to remove contaminating proteins and salts. The nucleic acid samples ($A_{260 \, nm}$ = 5 − 6) were mixed with DMSO (to 50 % DMSO final concentration). Samples were heated at 95 ℃ for 1 min . Identical samples mixed with DMSO were also prepared but without heating. Identical control samples mixed with an equal volume of nuclease-free water were also prepared (no heating). Triplicate biological replicates were performed for all samples analysed. Absorbance measurements were as follows.

A_0 = absorbance A_{260} of nucleic acid in H_2O

A_1 = absorbance A_{260} of nucleic acid in DMSO

A_2 = absorbance A_{260} of nucleic acid in DMSO + heat

$$\text{Hypochromicity factor (H)} = A_2 / A_0 \qquad (1)$$

$$A_2 / A_1 = (A_{260} \, \text{DMSO} + \text{heat}) / (A_{260} \, \text{DMSO}) \qquad (2)$$

$$A_1 / A_0 = (A_{260} \, \text{DMSO}) / (A_{260} \, H_2O) \qquad (3)$$

The nucleic acid extinction coefficient ($\mathcal{E}$) was determined from the sum of the individual nucleotide extinction coefficients as determined by [4].

$$\varepsilon_{sum} = n_A \varepsilon_A + n_G \varepsilon_G + n_C \varepsilon_C + n_U \varepsilon_U \text{ or } n_T \varepsilon_T, \qquad (4)$$

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where n_N= number of nucleotide, N, in the nucleic acid and corrected for the

hypochromicity using

$$\varepsilon_{nucleic\ acid} = (1/H) * \varepsilon_{sum} \qquad\qquad (5)$$

The concentration was determined using the Beer-Lambert equation using the predicted

and corrected extinction coefficient in conjunction with the sum of the mononucleoside

phosphates masses to determine the molecular mass of the nucleic acid.

Propagation of error

Propagation of error in all experimental ratios was determined by first calculating the

covariation of error and then the standard deviations. Standard deviations of the ratios

shown were determined by propagation of the standard deviations of the absorbance

values recorded in multiple measurements of aliquots of the same sample using UV

spectrophotometry.[19] $SD\ (a/b) = a/b\ \sqrt{((SD_a/a)^2 + (SD_b/b)^2)}.$ $\qquad\qquad (6)$

Agarose gel electrophoresis

1% agarose gels were used for gel electrophoresis. RNA loading dye 2x (NEB) was

added to RNA samples and loaded on the gel. 1X TAE buffer (40 mM Tris (pH 7.6), 20

mM acetic acid and 1 mM EDTA) was used to perform electrophoresis at 100 V for 45

mins. The agarose gels were pre-stained with ethidium bromide and images obtained

using a UV imaging system fitted with charge coupled device (CCD) camera

(Biospectrum® Multispectral Imaging System).

Nucleic acids used in this study

Nucleic acid	Sequence/Composition
NTPs	equimolar ATP/CTP/GTP/UTP
dNTPs	equimolar dATP/dCTP/dGTP/dTTP
15mer RNA oligo	CAAAAGUCCGUGAGA (A:6; C:3; G: 4; U: 2)
13mer DNA oligo	AGCTAGCTAGCTA (dA:4; dC:3; dG: 4; T: 2)
ssRNA (521 ntr)	(A:131; C:144; G102; U:144)
dsRNA (521 bp)	(A:275; C:246; G:246; U:275)
dsDNA (518 bp)	(dA:275; dC:243; dG:243; T:275)
dsRNA (686 bp)	(A:261; C:425; G:425; U:261)
dsRNA (481 bp)	(A:190; C:291; G:291; U:190)
ssRNA (600 ntr)*	(A:150; C;150; G150; U:150)
ssRNA (550 ntr)*	(A:150; C:100; G150; U:150)
ssDNA (600 ntr)*	(dA:150; dC;150; dG150; T:150)
ssDNA (550)*	(dA:150; dC;100; dG150; T:150)

Preparation of model/standard curves

A_2 / A_1 value of 1.105 would be obtained for RNA sample containing 50% $A_{260\ nm}$ of dsRNA and 50% $A_{260\ nm}$ of ssRNA where 1.0 and 1.21 is the A_2/A_1 values for pure ssRNA and dsRNA, respectively (50%*1.21 + 50%*1.0)

A model equation is therefore proposed:

A_2 / A_1 =1.21 (%Abs_{260} of dsRNA/100) + 1.0 (1 - %Abs_{260} of dsRNA/100)　　　　(7)

Or %dsRNA Abs260 = (A_2 / A_1 - 1)/0.0021.　　　　　　　　　　　　　　(8)

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By substituting a range of hypothetical %Abs$_{260}$ of dsRNA values in the equation, a range of hypothetical A_2 / A_1 values are obtained. Using these hypothetical data and plotting A_2 / A_1 values against %Abs$_{260}$ of dsRNA values we generate a model linear calibration curve. Similarly, a standard curve is obtained by plotting %Abs$_{260}$ of dsRNA against experimentally derived A_2 / A_1 values.

Concentration of dsRNA was determined using the %dsRNA Abs$_{260}$ as determined above in conjunction with the total A_{260} to calculate the corresponding A_{260} for dsRNA. Overall concentration was subsequently calculated using the value of 46.52 μg/mL/A_{260}:

Mass concentration = %dsRNA Abs$_{260}$/100 x Total RNA A_{260} x 46.52 μg/mL/$A_{260.}$ (9)

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Results and Discussion

1.0 Validation of a method using DMSO and heat to measure the hypochromicity of dsRNA

High DMSO concentrations (>75%) have previously been shown to disrupt the structure and stability of RNA[11,20] and DNA,[21,22] consistent with disruption of base-stacking interactions and increased flexibility. In order to perform hypochromicity measurements using DMSO in conjunction with thermal denaturation, a range of dsRNAs synthesized, in either *E. coli* or by *in vitro* transcription, were purified using a combination of RNase T1 and solid phase extraction to remove contaminating ssRNA and NTPs (see Figure S1). Following purification, the dsRNA (686 bp) was heated at 95 ℃ for 1 min in 50% DMSO and analysed using agarose gel electrophoresis (see Figure 1A). The results show the effective denaturation of the duplex dsRNA into the corresponding ssRNA preventing re-annealing of the dsRNA. In addition, decreased ethidium bromide fluorescence was observed in the corresponding ssRNA owing to reduced intercalation.

To further study the effects of DMSO, a range of dsRNA (521 bp and 481 bp) were incubated in the presence and absence of DMSO with and without heating (see Figure 1B). The results show a small relative reduction in the ethidium bromide fluorescence for dsRNA in the presence of DMSO without heating but no dissociation of strands (see Figure 1B). These results demonstrate that in the presence of 50% DMSO it is not sufficient to disrupt the duplex structures. However, it destabilizes base stacking

interactions, resulting in reduced intercalation of the ethidium bromide and subsequent fluorescence.

In addition to denaturation of dsRNA, the same approach was used to denature dsDNA (518 bp). dsDNA was generated by PCR, purified using solid phase extraction to remove ssDNA and dNTP impurities and analysed using IP RP HPLC (see Figure S2). The dsDNA was added to 50% DMSO including a thermal denaturing step to denature the dsDNA to its corresponding ssDNAs prior to analysis using gel electrophoresis (see Figure 1C). The results reveal that, unlike dsRNA, a proportion of dsDNA dissociates into ssDNA upon addition of 50% DMSO in the absence of heat. However, in the presence of 50% DMSO and thermal denaturation the dsDNA duplex completely dissociates into the corresponding ssDNA. These results highlight the difference in the stability of the dsDNA vs dsRNA duplex attributed to increased base-stacking interaction energy in the A-form conformation of dsRNA. We therefore used a range of DMSO concentrations to determine the appropriate concentration to disrupt the base stacking interactions without dissociating the duplex strands (see Figure 1D). The results show that using a lower % of DMSO (16%) reduced the amount of the dsDNA that was denatured but still caused strand separation upon heating.

1.1 Quantitative analysis of the hypochromicity of dsRNA

Following the successful demonstration that the addition of 50% DMSO with a short thermal denaturation step prevents re-annealing of the dsRNA, we used this method to determine the dsRNA hypochromicity in conjunction with UV spectrophotometry. A_{260}

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values were obtained in absence of DMSO (A_0) the presence of DMSO (A_1) and DMSO + heat (A_2) to denature the dsRNA to their corresponding ssRNAs. This enables an accurate A_{260} measurement of both the unstructured denatured nucleic acids and the intact non-denatured dsRNA (see Table 1). The hypochromicity factor (H) was determined by calculating the ratio of A_2/A_0. Values between 1.45-1.50 were obtained for a range of different dsRNA and 1.21 for ssRNA. The A_1/A_0 ratios observed for both ds and ssRNA were typically 1.22 demonstrating the reduction in single stranded base stacking interactions in ssRNA and a reduction in base stacking interactions in the duplex dsRNA with the addition of DMSO. Furthermore, heating of the ssRNA in the presence of DMSO does not change the A_{260} ratios demonstrating that no further change in hypochromicity is observed. However, heating of the dsRNA in the presence of DMSO causes a further increase in absorbance as the duplex strands are denatured to their corresponding single strands.

1.2 Quantitative analysis of the hypochromicity of structured yeast tRNAs

A high degree of secondary structure in specific RNA molecules can lead to a significant hypochromicity that is not accounted for in the standard methods to calculate extinction coefficients using nearest-neighbor effects, which results in a systematic underestimation of RNA concentrations. In addition, previous data have demonstrated that the thermal denaturation of riboswitch RNAs is not a convenient or reliable method for determining the UV absorbance without the hypochromic effect from base-pairing interactions [13,15]. For RNAs with a high degree of secondary structure thermal hydrolysis to the component mononucleoside phosphates has been used for quantification [15]. The

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ability of the developed method in this study to rapidly determine the hypochromicity of structured RNAs was analysed using phenylalanine tRNA and total tRNA from *S. cerevisae* (see Table 2). The results show hypochromicity factors of 1.22 and 1.21 for phenyl tRNA and total tRNA, similar to values obtained for larger ssRNA molecules. In addition, similar to ssRNA, further heating of the tRNAs in the presence of DMSO does not significantly change the A_{260} ratios, demonstrating that no further change in hypochromicity is observed. These results demonstrate that for structured RNAs such as tRNAs, the addition of 50% DMSO is sufficient to destabilise their base stacking interactions and intramolecular folding.

1.3 Quantitative analysis of the hypochromicity of dsDNA, oligonucleotides and oligoribonucleotides

The hypochromicity factor (A_2/A_0) for dsDNA was determined to be 1.56 and is higher than that obtained for dsRNA (see Table 2). It is noteworthy that the A_1/A_0 ratio is higher than that obtained for dsRNA owing to the significant ssDNA generated upon addition of 50% DMSO. In addition to the analysis of large ss/ds nucleic acids typically > 400 nt/bp, hypochromicity measurements of short oligonucleotides (<20 nts) were also obtained (see Table 2). The results show that similar hypochromicity values of 1.21 were obtained for oligonucleotides/oligoribonucleotides when compared to larger ssRNA and ssDNA molecules. This suggests that intramolecular interactions/ss base stacking interactions in short oligonucleotides are similar to those in large ssDNA and ssRNA. Furthermore, hypochromicity factors for NTPs and dNTPs were also determined (see

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Table 2). The results show no change in $A_{260 \, nm}$ were observed demonstrating that there

is no alteration in the extinction coefficients in the presence of DMSO and as expected

they do not exhibit hypochromicity upon heating in DMSO.

2.0 Determination of nucleic acid extinction coefficients and concentration using hypochromicity measurements

In this study, we have used the measurements of hypochromicity outlined previously to

accurately determine the overall extinction coefficient and mass concentration/A_{260} for a

range of nucleic acids. In each of the nucleic acids analysed the base sequence and

composition is known, therefore we have used the sum of the monomer extinction

coefficients as determined in [4] to compare the calculated and measured extinction

coefficients based on the hypochromicity measurements (see Table 3). In addition,

using the sum of the mononucleoside phosphate masses we have determined the mass

concentration/A_{260} in conjunction with the calculated and measured extinction

coefficients (see Table 3).

The results obtained in Table III demonstrate that for a number of different dsRNA

sequences typical values of 46.18-47.29 (median=46.52) µg/ml/A_{260}/ were obtained. To

our knowledge we believe this is the first time an accurate value for the calculation of

the concentration of dsRNA directly from A_{260} measurements has been experimentally

obtained. For larger ssRNAs a value of 37.80 µg/ml/A_{260} (equimolar mononucleotide)

was obtained which is lower than the typically reported value often used for RNA 40

µg/ml/A_{260}. For ssRNA oligoribonucleotides (13 mer) a value of 34.85 µg/ml/A_{260} was obtained. For dsDNA (dA:275; dC:243; dG:243; T:275) a value of 46.25 µg/ml/A_{260} was determined compared to previously reported values typically 50 µg/ml/A_{260}.[2]

The results show that for ssDNA (13 mer) a typical value of 34.53 µg/ml/A_{260} and ssDNA (550 ntr) 34.73 µg/ml/A_{260} was obtained. For ssRNA and ssDNA oligonucleotides containing equimolar base composition a typical value of 37.80 and 35.70 µg/ml/A_{260} was predicted using this approach. For structured tRNAs values of 37.80 and 37.18 µg/ml/A_{260} were obtained taking into account the hypochromicity measurement.

3.0 Quantification of ds and ss nucleic acids in complex mixtures

Previous results showed that in the presence of 50% DMSO further heating of ssRNA resulted in no hyperchromic effect ($A_2/A_1 = 1.0$) whilst heating of dsRNA in 50% DMSO resulted in a hyperchromic effect ($A_2/A_1 = 1.2$). Therefore, it is proposed that a sample containing a mixture of dsRNA and ssRNA upon heating in DMSO, will have a hyperchromic effect between 1.0 to 1.2 depending on the relative proportion of dsRNA in the sample. We can therefore generate a model linear curve of (A_2/A_1) against % dsRNA values (see Figure S3). To experimentally validate the model, standards containing a range of known proportions of dsRNA relative to ssRNA were prepared (see Figure S4) and A_2/A_1 ratios determined as previously described and plotted against the relative % of dsRNA (see Figure 2A). The results show the expected linear relationship between the relative % of dsRNA and A_2/A_1 ratio.

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We used this approach to determine an unknown percentage and concentration of dsRNA in a complex mixture (see Figure 2B, Figure 3). To demonstrate the accuracy of method, the relative proportion and concentration of total dsRNA and dsRNA were determined as 24.4% (281.1 ng/μL) and 49.1% (565.5 ng/μL) for known RNA standards containing 25% and 50% dsRNA respectively. Samples containing an unknown quantity of dsRNA were determined to be 15.2% (175.7 ng/μL) dsRNA.

Similar to the method described above the same approach can be used to measure the amount of dsDNA present in complex ds/ss DNA mixtures using the linear relationship between the A_2/A_1 ratio and % dsDNA absorbance. For a known DNA standard containing 45% dsDNA the relative proportion of total dsDNA was determined as 44.75% (see Figure 2C).

It should be noted that although a small concentration of the dsDNA is denatured at 16% DMSO, this does not affect the determination of the relative proportion dsDNA; the hypochromicity due to the denatured DNA is reflected in the A_1/A_0 ratio (1.37) and accounted for in the equation ($A_2 / A_1 = 0.0036$ (dsDNA Abs_{260}) + 1.0069). This provides a rapid, high throughput quantitative and qualitative approach to analysing the amount of dsRNA/DNA present in all mixed DNA or RNA nucleic acid samples. No separation or purification of the dsRNA/DNA is required prior to accurate quantification. To our knowledge this is the first time UV spectrophotometry has been used for accurate quantification of duplex structures in mixed ds/ss nucleic acid solutions.

Conclusions

UV spectrophotometry has been used to rapidly determine the hypochromicity of a wide range of nucleic acids including ssDNA/RNA, oligonucleotides, structured RNA and dsRNA. We have utilised the chemical denaturant dimethyl sulfoxide in conjunction with short thermal denaturation which prevents renaturation of the duplex nucleic acids (ds DNA/RNA) to measure the absorbance of both the unstructured and structured nucleic acids. This approach does not require prolonged, elevated temperatures, which may cause partial hydrolysis of RNA or incomplete denaturation of the dsRNA. In addition, this method is not affected by changes in the extinction coefficients of nucleic acids with temperature, as all A_{260} measurements are performed at room temperature. Using this approach, we have used the measurements of hypochromicity outlined previously to accurately determine the overall extinction coefficient and mass concentration/A_{260} for a range of nucleic acids. We have for the first time determined a median value of 46.52 µg/ml/A_{260} for the quantification of dsRNA using UV spectrophotometry, enabling the accurate determination of the relative proportion of duplex nucleic acids in mixed ds/ss nucleic acid solutions and demonstrating significant advantages over current methods. This provides a rapid, high throughput quantitative and qualitative approach to analysing the amount of dsRNA present in all RNA samples. No separation or purification of the dsRNA is required prior to accurate quantification. To our knowledge this is the first time UV spectrophotometry has been used for accurate quantification of duplex structures in mixed ds/ss nucleic acid solutions.

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Acknowledgements

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Figure Legends:

Figure 1. Agarose gel electrophoresis analysis of the effects of DMSO on dsRNA (A) 50% DMSO was added to purified dsRNA (686 bp). Thermal denaturation was performed at 95 ºC for 1 min. B) 50% DMSO was added to purified dsRNA (481 bp and 518 bp) dsRNA with and without additional thermal denaturation at 95 ºC for 1 min. C) Agarose gel electrophoresis analysis of the effects of 50% DMSO on dsDNA (518 bp). D) A range of DMSO concentrations were added to dsDNA (518 bp and 823 bp) with and without additional thermal denaturation at 95 ºC for 1 min.

Figure 2. Quantification of ds RNA/DNA in nucleic acid mixtures using spectrophotometry. (A) Standard curve generated using mixtures of dsRNA (521 bp) and ssRNA (521 nt) standards. Following the experimental determination of the A_2 and A_1 values, the ratio A_2/A_1 of the corresponding mixtures were plotted against % dsRNA and the equation obtained is given by %dsRNA Abs_{260} = (A_2/A_1 - 1.0051)/0.002). (B) Standard curve generated using mixtures of 521 bp dsRNA and ssRNA (*E. coli* total RNA). A_2/A_1 ratios of the corresponding mixtures were plotted against % dsRNA and the equation is given by %dsRNA Abs_{260} = (A_2/A_1 - 1.0038)/0.0021). (C) Standard curve generated using mixtures of dsDNA and ssDNA (oligonucleotides). Following the experimental determination of the A_2 and A_1 values for dsDNA, the ratio A_2/A_1 were plotted against percentage dsDNA and the equation is given by %dsDNA Abs_{260} = (A_2/A_1 -1.0069)/0.0036).

Figure 3. IP RP HPLC analysis of nucleic acid mixtures containing varying proportions of dsRNA (521 bp) and ssRNA (*E. coli* total RNA). (A) IP RP HPLC analysis (using gradient 1) of a mixture containing 25% dsRNA (521 bp) (based on A_{260}). The A_2/A_1 ratio for this nucleic acid mixture predicted 24.38% dsRNA, corresponding to a dsRNA concentration of 281.08 ng/µl (B) IP RP HPLC analysis (using gradient 1) of a mixture containing 50% dsRNA (521 bp) (based on A_{260}). The A_2/A_1 ratio for this complex mixture predicted 49.05% dsRNA corresponding to a dsRNA concentration of 565.52 ng/µL. (C) IP RP HPLC analysis (using gradient 2) of a complex mixture containing an unknown amount of dsRNA (481 bp). The A_2/A_1 ratio predicted 15.24% dsRNA corresponding to a concentration of 175.71 ng/µL.

Supporting Information

IP RP HPLC analysis of experimental RNA and DNA samples, model linear curves for the quantification of ds and ssRNA/DNA in complex nucleic acid mixtures, IP RP HPLC analysis of nucleic acid mixtures containing varying proportions of dsRNA and *E. coli* total RNA.

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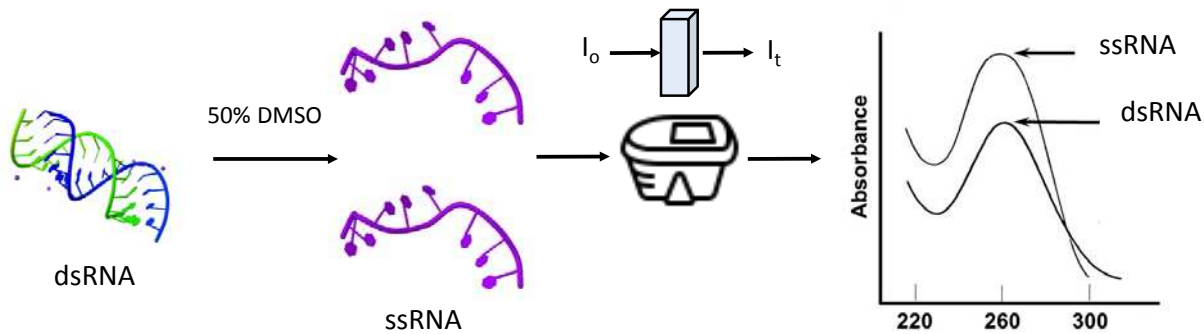46
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TOC only

dsRNA

50% DMSO

ssRNA

I_o → I_t

Absorbance

ssRNA

dsRNA

220   260   300

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Figure 1

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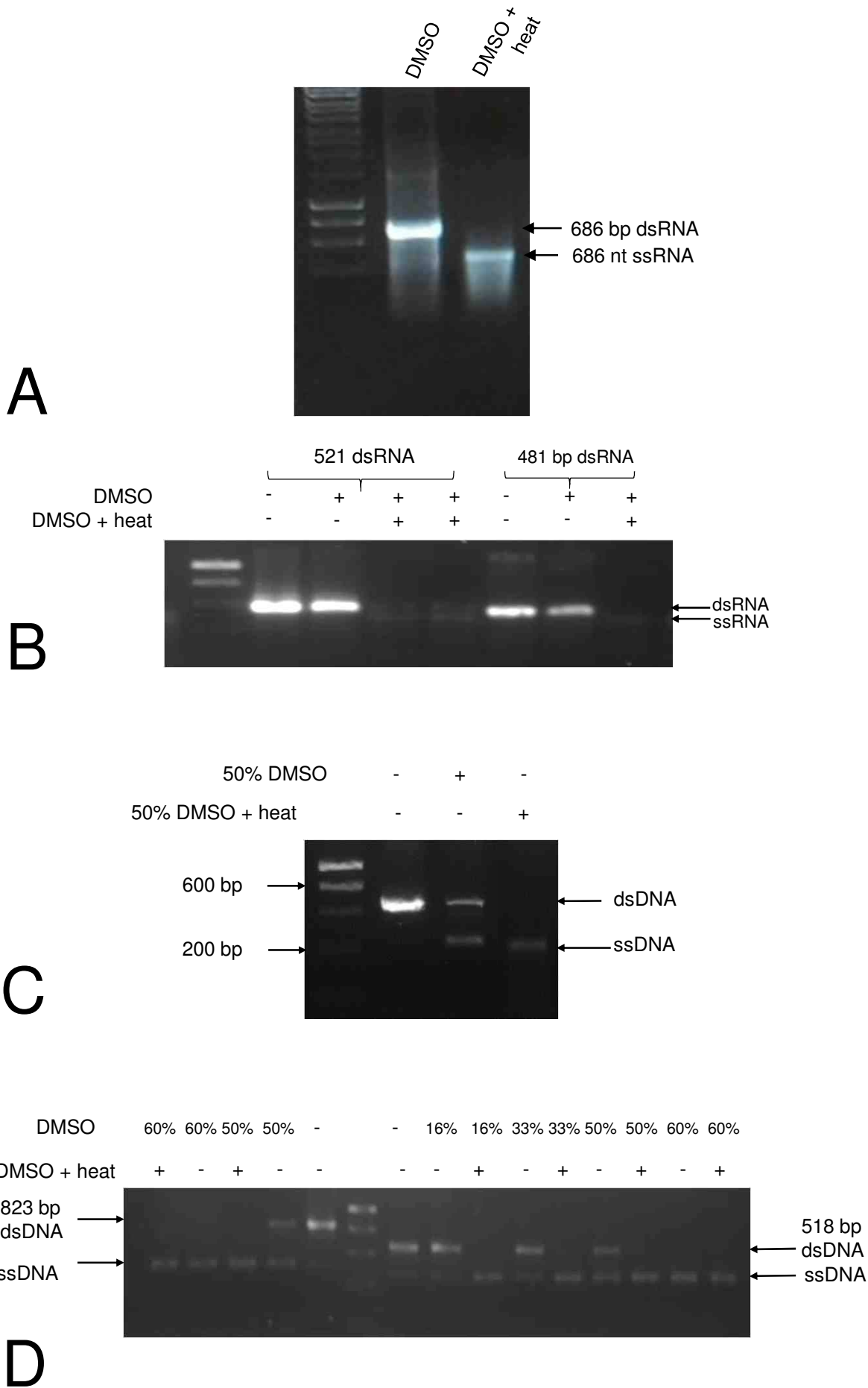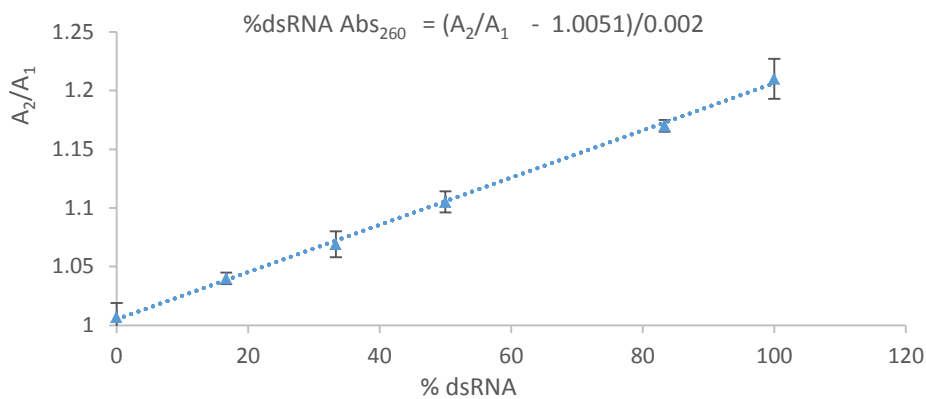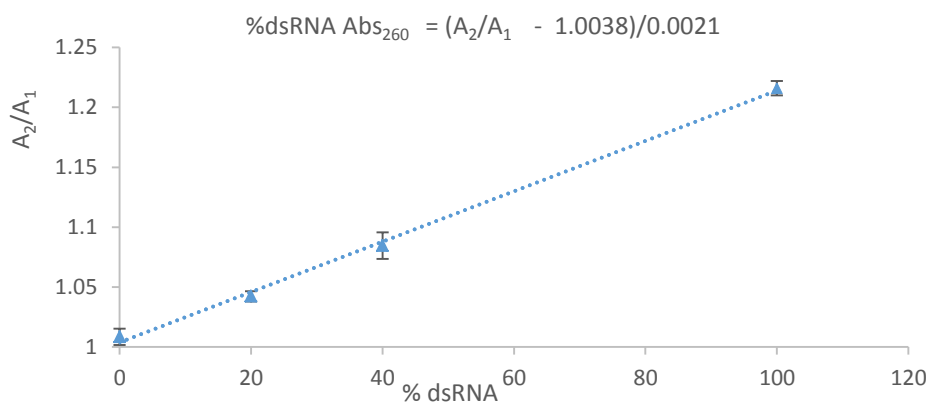51
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A

%dsRNA Abs$_{260}$ = (A$_2$/A$_1$ − 1.0051)/0.002

B

%dsRNA Abs$_{260}$ = (A$_2$/A$_1$ − 1.0038)/0.0021
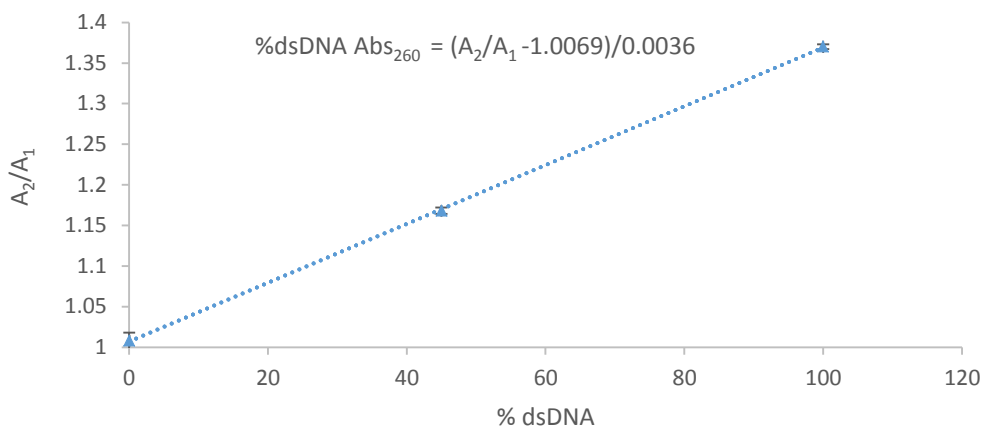
C

%dsDNA Abs$_{260}$ = (A$_2$/A$_1$ −1.0069)/0.0036

Figure 2

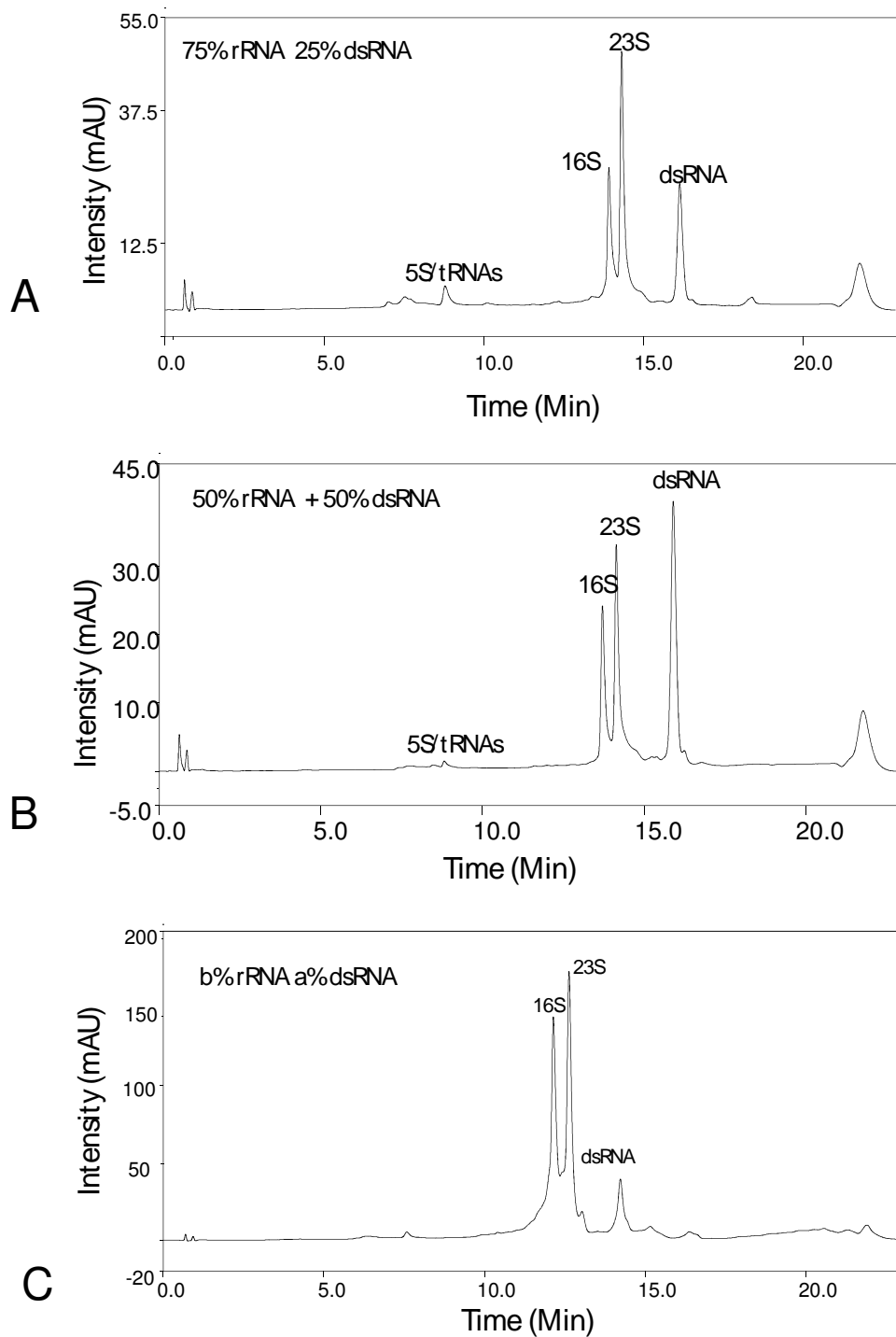Figure 3

Table 1. Hypochromicity measurements of ssRNA and dsRNA. A_{260} values were obtained in the absence of DMSO (A_0), the presence of DMSO (A_1) and DMSO + heat (A_2) to denature the dsRNA to their corresponding ssRNAs. The hypochromicity factor (H) was determined by calculating the ratio A_2/A_0. Absorbance values are shown as means (n=3) ± propagated S.D.

Nucleic Acid A_{260}	A_1/A_0	A_2/A_0	A_2/A_1
dsRNA (521 bps)	1.24±0.005	1.50±0.007	1.21±0.005
dsRNA (481 bps)	1.24±0.004	1.48±0.006	1.20±0.005
dsRNA (686 bps)	1.21±0.003	1.45±0.004	1.20±0.004
	A_1/A_0	A_2/A_0	A_2/A_1
ssRNA A (521 nt)	1.22±0.005	1.22±0.006	1.00±0.005
ssRNA B (521 nt)	1.22±0.006	1.23±0.006	1.00±0.005
ssRNA C (521 nt)	1.22±0.007	1.21±0.008	1.01±0.008
Average	1.22±0.010	1.22±0.012	1.00±0.011
$\dfrac{\text{dsRNA } A_2/A_0}{\text{ssRNA } A_2/A_0}$		1.48/1.22 = 1.21	

Table 2. A summary of the ratios of the A_{260} measurements A_{260} values were obtained in absence of DMSO (A_0) the presence of DMSO (A_1) and DMSO + heat (A_2) to denature the dsRNA to their corresponding ssRNAs. This enables an accurate A_{260nm} measurement of both the unstructured denatured nucleic acids and the intact non-denatured dsRNA (see Table 1). The hypochromicity factor (H) was determined by calculating the ratio of A_2/A_0. Absorbance values are shown as means (n=3) ± propagated S.D.

Sample	A_1/A_0	A_2/A_0	A_2/A_1
NTPs	1.00±0.002	1.00±0.002	1.00±0.002
dNTPs	1.00±0.004	1.00±0.005	1.00±0.004
ssRNA oligoribonucleotide (15 mer)	1.21±0.003	1.21±0.007	0.99±0.006
ssDNA oligonucleotide (13 mer)	1.21±0.007	1.21±0.005	1.00±0.007
ssRNA (521 nt)	1.22±0.010	1.22±0.012	1.00±0.011
dsRNA (521 bp)	1.24±0.005	1.50±0.007	1.21±0.005
dsRNA (481bp)	1.24±0.004	1.48±0.006	1.20±0.005
dsRNA (686 bp)	1.21±0.003	1.45±0.004	1.20±0.004
dsDNA (518 bp)	1.40±0.004	1.56±0.002	1.11±0.003
Phenylalanine tRNA (*S. cerevisae*)	1.22±0.003	1.22±0.003	1.00±0.003
Total tRNA (*S. cerevisae*)	1.20±0.003	1.21±0.003	1.00±0.001

Table 3. Determination of the extinction coefficient (ε) and concentration of nucleic acids using hypochromicity factors (H) in conjunction with A_{260} measurements. The nucleic acid extinction coefficient (ε) was determined from the sum of the individual nucleotide extinction coefficients as determined by (4). The concentration was determined using the overall predicted extinction coefficient in conjunction with the sum of the mononucleoside phosphate masses to determine the molecular mass of the nucleic acid. * The calculated nucleic acid extinction coefficient and concentration determined using the hypochromicity factors (H).

Nucleic acid	Sequence	(H)	ε $(mM^{-1} cm^{-1})$	ε^* $(mM^{-1} cm^{-1})$	Conc (μg /ml/A_{260})	Conc* (μg /ml/A_{260})
ssRNA (600 nt)	(A:150;C:150; G:150;U:150)	1.22	6574.50	5388.93	30.98	37.80
ssRNA (550 nt)	(A:150;C:100; G:150;U:150)	1.22	6221.00	5099.18	30.14	36.77
ssDNA (600 nt)	(dA:150;dC;150; dG:150;T:150)	1.21	6435.00	5318.18	29.504	35.70
ssDNA (550 nt)	(dA:150;dC:150; dG:150;T:150)	1.21	6080.00	5024.79	28.702	34.73
ssRNA (15 nt)	CAAAAGUCCGUGAGA	1.21	178.90	147.91	28.80	34.85
ssDNA (13 nt)	AGCTAGCTAGCTA	1.21	147.40	121.80	28.53	34.53
ssRNA (521 nt)	(A:131; C:144; G102; U:144)	1.22	5608.90	4597.46	31.36	38.27
dsRNA (521 bp)	(A:275; C:246; G:246; U:275)	1.50	11497.90	7665.27	30.78	46.18
dsRNA (481 bp)	(A:190; C:291; G:291; U:190)	1.48	10261.85	6933.68	31.95	47.29
dsRNA (686 bp)	(A:261; C:425; G:425; U:261)	1.45	14580.23	10055.33	32.08	46.52
dsDNA (518 bp)	(dA:275; dC:243; dG:243; T:275)	1.56	11180.54	7167.01	29.65	46.25